A Comparison of Some Potential Indicators of Desiccation-tolerance in 2 Poikilochlorophyllous *Xerophyta* species and Homiochlorophyllous *Craterostigma wilmsii.*

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Botany Honours Thesis
University of Cape Town
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2002

Contents

Abstract

Three possible indicators of desiccation-tolerance in angiosperms: sucrose accumulation and hexose sugar flux, an increase in hexokinase activity, and accumulation of LEA-like proteins, were investigated and compared during dehydration between two moncotyledonous poikilochlorophyllous resurrection angiosperm species; *Xerophyta schlecterii* and *Xerophyta humilis,* and a dicotyledonous homiochlorophyllous resurrection species, *Craterostigma wilmsii.* Comparisons were also made with *Xerophyta viscosa,* using data from Whittaker *et al*, (2001). Soluble sugar concentrations and hexokinase activities were also examined during rehydration of the three species.

Sucrose was accumulated during dehydration in all species examined. However, *C.wilmsii* had completed sucrose accumulation by the intermediate stages of drying, whereas *X.humilis* exhibited a late accumulation. It was not clear if *X.schlecterii* exhibited late sucrose accumulation due to high standard deviations in the results. *C.wilmsii* accumulated higher sucrose levels than the two poikilochlorophyllous species. During rehydration the sucrose concentrations dropped in all three species, and was probably being used as an energy source for the resumption of metabolism. Glucose and fructose concentrations were relatively constant during dehydration in all three species, and did not appear to be contributing greatly to sucrose accumulation. This was further confirmed by the low levels of activity of hexokinase found in all three species during dehydration and rehydration, which suggests that the channelling of glucose and fructose into sucrose biosynthesis is not being upregulated during dehydration. *C.wilmsii* had an increased level of

hexokinase late in dehydration, that may have been in preparation for the rapid rehydration experienced by this homiochlorophyllous species, where hexokinase would need to rapidly channel glucose and fructose into metabolism. There was not an increase in activity at the period of highest sucrose accumulation in this species. The activity of hexokinase was relatively constant throughout dehydration and rehydration for *X.schlecterii,* as it was for *X.humilis.* Glucose and fructose levels dropped slightly in *C.wilmsii* during rehydration but remained relatively constant in the two poikilochlorophyllous species.

The presence of LEA-like proteins was found in all three species, although the bands were very faint for *X.viscosa* and *X.humilis.* In *X.schlecterii* the bands were clear in the dehydrated leaf sample, but were faint or absent in the hydrated sample. It is possible that the proteins in this species were upregulated or induced by drying. A protein common to all three species in dehydrated and hydrated samples was observed between the 22.5 kDa and the 15.3 kDa molecular markers.

Overall, there were differences in the timing and amount of sucrose accumulation between the homiochlorophyllous and poikilochlorophyllous species, but all three were similar in that they did not appear to rely on glucose and fructose for sucrose biosynthesis and hence did not upregulate hexokinase activity during drying. This contrasts with *X.viscosa,* which exhibited increased hexokinase. So although there are differences in the patterns of accumulation, all three species tested accumulated sucrose during drying and hydrolysed it during rehydration, none of them appeared to upregulate hexokinase during drying, and all species tested had LEA-like proteins present in hydrated and dehydrated tissue.

Introduction

Desiccation-tolerance is a phenomenon that has been observed in orthodox seeds, pollen, lower plants such as bryophytes, algae, and lichens, and in some higher angiosperms. Poikilohydrous or resurrection plants are able to survive desiccation, and can lose up to 95% of their initial water content and still become metabolically active if water becomes available again (Bernacchia *et al,* 1996, as cited by Scott, 2000). Generally lower plants survive desiccation by the repair of damage during rehydration. Higher plants generally prevent damage during dehydration by protection mechanisms that minimise damage (Farrant, 2000). These plants shut down metabolism during dehydration and survive in a stasis until rehydration. After rehydration they can resume cellular functioning, with minimal tissue damage (Scott, 2000).

The challenges faced by a poikilohydrous desiccation tolerant species while dehydrating are: (1) to minimise mechanical damage to the cell due to turgor loss; (2) to try and maintain the integrity of macromolecules and membranes by replacement of hydrogen bonds usually formed by water with these structures; and (3), to minimise free radical and toxin damage (Bewley, 1979, Sherwin and Farrant, 1996 and Farrant *et al,* 2002 (unpublished)). Light energy harvested by chlorophyll cannot be dissipated through photosynthesis during conditions of low cellular water and this leads to the formation of reactive oxygen species or free radicals, which damage cell contents (Sherwin and Farrant, 1998, Farrant, 2002). During rehydration the cell must be able to rapidly resume respiration and again minimise mechanical damage that may occur due to rapid changes in cell turgor. In general homiochlorophyllous species (species

that maintain the structural integrity of their chloroplasts during dehydration and mask the chlorophyll to prevent photosynthesis, by producing reflective pigments such as anthocyanins or by curling of leaves (Farrant, 2000)) such as *Craterostigma plantagineum* and *Craterostigma wilmsii* (Scrophulariaciae), *Myrothamnus flabellifolius* and *Sporobolus stapfianus* (Poacae) (partially homiochlorophyllous) recover more quickly, as they are able to immediately resume photosynthesis, whereas poikilochlorophyllous species such as *Xerophyta viscosa, X.schelcterii* and *X.humilis* (Velloziacae) which dissasemble their thylakoid membranes during desiccation, have to rebuild their chloroplasts before photosynthesis can resume (Sherwin and Farrant, 1996).

A common mechanism of desiccation-tolerance is osmolyte accumulation in the form of sugars such as sucrose, and proteins. Hexokinase is an important enzyme involved in metabolism and biosynthesis, including the synthesis of sucrose, and has been found to have had increased activity in some desiccation-tolerant species during dehydration (Whittaker *et al,* 2001).

Sugars

Sugars are known to be accumulated by all desiccation tolerant species studied so far during drying, in both plants and animals (Scott, 2000, Crowe L M, 2002). Lower plants and animals tend to accumulate trehalose, whereas higher plants tend to accumulate sucrose. There are two hypotheses as to the role of sucrose accumulation during drying (Vertucci and Farrant, 1995). Firstly, it is thought to be a compatible

solute, in that in the absence of water, sucrose will replace hydrogen bonds in membranes and proteins that were previously maintained by water (Crowe *et al,* 1986. as cited by Scott, 2000). Sucrose is also associated with vitrification or glass formation, which is thought to put a stasis on metabolism while the plant is in a dry state (Crowe *et al,* 1998, as cited by Scott, 2000). It is thought that to avoid sucrose crystallisation, glasses also include trisaccharides such as raffinose and stachyose, which, due to an extra galactose group, only partially fit into the growing sugar crystal (Sun and Leopold, 1997, as cited by Crowe, 2002)

In *Craterostigma* species it is thought that a major source for sucrose accumulation during drying is 2-octulose, which is a photosynthetic storage reserve (Norwood *et al*, 2000), although it has been shown that during dehydration in *C.plantagineum* there is no fall in 2-octulose concentration in the leaves although there is significant sucrose accumulation (Norwood *et al,* 1999). It is likely that 2-octulose is not the only source of sucrose during dehydration in this species, but rather a redistribution of carbohydrates between the different plant organs (e.g. 2-octulose and starch), as well as root reserves and products translocated from old senescing leaves all contribute to this accumulation (Norwood *et al,* 1999). It is not known if 2-octulose is major source of sucrose in *Xerophyta* species. Other sources may include any stored starch in chloroplasts, but this would only be important in the first stages of drying because many of these desiccation tolerant plants shut down photosynthesis while drying to prevent cell damage from reactive oxygen species formation. Stored starch would therefore be used up early in the drying response, but these plants often continue to accumulate sucrose quite late in the drying stages.

Xerophyta species may have another source of sucrose. It is also possible that stored fructans (which are known to accumulate in grass species under conditions of high light or low temperature) (Salisbury and Ross, 1992) are a source of substrate for sucrose synthesis in the *Xerophyta* species. Fructan accumulation has been associated with increasing drought tolerance in wheat, another monocotyledonous species (Kerepesi *et al*, 2002).

It could be possible that glucose and fructose in the cell can also contribute to the sucrose accumulation. Levels of free glucose and fructose per sê are not important in the synthesis of sucrose; rather the levels of the phosporylated forms of these hexose sugars are important (glucose-6-phosphate and fructose-6-phosphate) (Salisbury and Ross, 1992). The enzyme, hexokinase that catalyses the phosphorylation of both fructose and glucose will then play an important role in sucrose accumulation if theses hexoses are being directly used as sources for sucrose synthesis.

Levels of glucose and fructose have been reported to remain relatively constant or to peak early in dehydration (possibly due to starch breakdown early in dehydration) but to decline in the later stages of drying in *Sporobolus stapfianus* and *Xerophyta viscosa* (Whittaker *et al,* 2001). This decline may indicate that some glucose and fructose are being utilised for sucrose synthesis in these species.

Hexokinase

Increasing hexokinase activity has been associated with hydrolytic starch degradation in tobacco (Häusler *et al,* 1998, as cited by Whittaker *et al,* 2001), which is thought to occur in the early and intermediate stages of dehydration to supply substrate for sucrose accumulation.

Respiration is maintained in the early and intermediate stages of drying (Farrant 2001). Sucrose is the most common supplier of glucose and fructose during glycolysis (Salisbury and Ross, 1992), but during later stages of drying glycolysis decreases, and sucrose accumulates, so it is possible that free glucose and fructose in the cells are being phosphorylated by hexokinase and channelled into sucrose synthesis instead of glycolysis. It is thought that the main importance of hexokinase during desiccation in the tolerant plants is to ensure that the amount of glucose and fructose being phosphorylated and therefore fed into metabolism exceeds their production (Whittaker *et al,* 2001).

Acid invertase activity (hydrolysis of sucrose to fructose and glucose) was found to be maintained during dehydration in *S.stapfianus* and *X.viscosa* (possibly in preparation for rehydration so that rapid hydrolysis of sucrose may occur to refuel metabolism), so hexokinase may be upregulated during drying to counteract this continued breakdown of sucrose by acid invertase (Whittaker *et al*, 2001). An increase in hexokinase activity during dehydration was reported by Whittaker *et al* (2001) in *S.stapfianus* and in *X.viscosa.*

Hexokinase is also thought to be a sugar sensor in sugar signal cascades. The phosphorylating step is thought to be the trigger in this sensing mechanism. During dehydration this may be important. If the levels of sugars that trigger hexokinase-sensing change, the induction of certain genes may occur (Loreti *et al,* 2001). High sucrose levels could possibly trigger genes involved in desiccation tolerance.

LEA-Like proteins

These proteins have been found to accumulate in response to many stresses in plants, as well as in fungi and yeast. They are highly hydrophilic due to their high glycine content and are not denatured by heat, even after exposure to 100° C. They are called LEA-like proteins as they were first reported to accumulate in abundance during the late stages of embryogenesis and were isolated from dehydrated orthodox seeds. Some of these proteins have also been named heat shock proteins (HSP's) or dehydrins. Six groups of LEA-like proteins have been identified and classified according to amino acid sequence (Dure *et al*, 1989, as cited by Sales *et al,* 2000). The dehydrin family of proteins has a highly conserved consensus sequence: KIKEKLPG, which is not present in any other proteins (Bracale *et al,* 1997). These LEA-like proteins are accumulated by many plant species in response to many abiotic stresses, including heat and osmotic stress. (Xiong and Zhu, 2002). They include chaperonins, which may prevent denaturation of heat-sensitive or dehydration-sensitive proteins by binding and maintaining hydrogen bonds that were maintained previously by water and in this way helping the molecule to return to its correct tertiary structure, much like compatible solutes such as sucrose (Blackman *et al,*

1991, as cited by Bracale *et al,* 1997). They also prevent the aggregation of the partially unfolded proteins, and may be involved in ion sequesterisation (Sales *et al,* 2000). They may also be involved in subcellular stabilisation (Sherwin and Farrant, 1996, Bracale *et al,* 1997). Stabilisation of the DNA molecule may also be a role during stress; these proteins have been found in nuclei in pea and tomato (Bracale *et al,* 1997).

It was found by Wolkers *et al* (2001), that a LEA protein isolated from *Typha latifolia* pollen has an unordered conformation in solution. During drying the protein assumes a conformation that is dependent on the rate of drying (with an α-helical conformation being assumed during rapid dehydration, and a mixed α-helix and β-sheet conformation during slow drying), and on the presence of sucrose. The α-helix conformation, which has been linked to LEA protein functioning, is only maintained in the presence of sucrose, which occurs in high amounts in the glassy state in the dehydrated pollen. When this protein was added to an *in vitro* sucrose glass, the strength of the hydrogen bonding and therefore the stability of the glass was improved. This suggests that LEA proteins and sugars may act together to form glasses, with the LEA proteins acting as links or anchors to add stability to cellular structures, to prevent denaturation of macromolecules, and to slow down the diffusion of free radicals (Wolkers *et al,* 2001).

Desiccation tolerant plants may produce specific LEA-like or heat-soluble proteins during dehydration which aid in the tolerance of water loss, or these proteins may be considerably up-regulated during desiccation stress.

Objectives

One objective of this study was to compare osmolyte accumulation (sucrose and LEA proteins) and hexose sugar flux in the species *Craterostigma wilmsii, Xerophyta schlecterii* and *Xerophyta humilis* during dehydration (drawing comparisons with *X.viscosa*), and to discover whether the enzyme hexokinase, that was found to have increased activity during dehydration in *Sporobolus stapfianus* and *Xerophyta viscosa* by Whittaker *et al,* (2001) plays an important role in the sucrose accumulation in any of these three species.

Another objective was to compare sucrose and hexose sugar concentrations in the three species during rehydration, and to see if hexokinase activity was upregulated to phosphorylate glucose and fructose into metabolism in these species, downregulated, or remained constant.

Materials and Methods

Plant Materials

One tray of *Craterostigma wilmsii, Xerophyta viscosa* and *Xerophyta schlecterii* and two trays of *Xerophyta humilis* were used. Trays were kept in a plant growth room with a light intensity of 45μmol $m^{-2}s^{-2}$, temperature of 25^0C, relative humidity between 50-70% and a 14 hour light cycle from 6am to 8 pm.

Dehydration and Rehydration

Trays were dehydrated by withholding water from the plants. Leaf samples were taken at intervals during drying for sugar, hexokinase and heat-soluble protein analyses, aiming for leaf relative water contents (RWC) of 100%, 70-50%, 30% and dry (below 10% RWC). For the heat-soluble protein analyses, only 100% RWC and dry samples were needed. Leaf samples were taken at approximately 11am unless a certain % RWC was reached much later. For *X. viscosa,* only a 100% RWC and a dry sample were required as only heat-soluble protein analysis was performed on this plant, so the plant was left until dry. It was found that plants growing in clay soils dried down much more slowly than those in loamy soils, so sampling times were adjusted according to soil type in which the plants were growing.

After plants had been dehydrated for one month, they were rewatered, and soil was kept constantly damp thereafter. Leaf samples were taken after 20 hours from initial rehydration, and after 92 hours, when the plants had fully rehydrated.

Leaf samples were cut, placed in foil and immediately immersed in liquid nitrogen, then stored in at –80°C.

% RWC was calculated as:

$$\%RWC = \frac{\text{(sample wet mass - dry mass) (100)}}{\text{Full turgor weight – full turgor dry weight}}$$

Hexokinase extraction and measurements

For each sample point two replicates of 1-4 leaves were extracted to give a sample of 0.05-0.1g each. Not enough replicates were used to perform statistical analyses on any of the experiments conducted.

Number of leaves used per replicate depended on leaf size of each species. Leaves were ground with liquid nitrogen once an equivalent mass of insoluble polyvinylpolypyrrolidone (PVP) was added to the sample (to protect the enzyme from phenolic compounds). The method of extraction is as in Whittaker *et al*, (2001). Ice cold extraction buffer consisting of 50mM KH_2PO_4 (pH 7.5), 4mM $MgCl_2$, 1mM EDTA, 10% glycerol and 5mM dithiothreitol (DTT) was added to the ground sample in a tissue sample mass to extraction buffer ratio of 1:20/30 for hydrated samples and 1:50 for dehydrated samples. Samples were then centrifuged for 20 minutes at 10 000rpm. 5ml Sephadex G-25 columns (particle size 50 – 150µm) were washed with water, equilibrated with extraction buffer, and centrifuged for 3min at 2 000 rpm.

2mls of supernatant from the tissue extract was then passed through these columns and centrifuged again, to remove phenolic compounds from the extracts.

Hexokinase activity was then assayed for in the extracts using a Beckman DU 650 spectrophotometer. The reaction mix contained 100mM KH_2PO_4 (pH 7.5), 2mM $MgCl_2$, 1mM EDTA, 15 mM KCl, 0.4mM NAD, 1mM ATP, 1.0 IU glucose-6-phosphate dehydrogenase (G6PDH), 1 IU phosphoglucose isomerase (PGI), 200ul of extract, 155µl H_2O and 5mM glucose as the enzyme substrate. Blanks contained all of the above reagents except for glucose. The rate of conversion of NAD to NADH was measured at 340nm over 12-16 minutes, and enzyme activity was calculated as µmol min^{-1} g^{-1} DW. Each extract was assayed twice to give a total of 4 replicates per plant at each % RWC.

Soluble sugar analysis

For each analysis two replicates were used, each of 1-4 leaves from different plants, depending on leaf size. Tissue was ground in liquid nitrogen. An extraction buffer consisting of 100mM NaOH in 50% v/v ethanol was added to the tissue in a tissue to buffer ratio of 1:20 for hydrated samples and 1:80 for dehydrated samples. Samples were incubated in the buffer on ice for ten minutes, then neutralised to pH 7.0-pH8.0 by the addition of 100mM HEPES in 1M acetic acid. Samples were then centrifuged at 15 000rpm for 15 minutes. Supernatants were stored and pellets were re-extracted. The two supernatants were then combined.

The soluble sugar content of each extract was measured enzymatically using a sugar food analysis kit. The method is as described in (Lang and Michal, 1974, as cited by Whittaker *et al,* 2001). Sugar concentrations were expressed as μmol g^{-1} DW.

Heat-soluble proteins extraction

Tissue samples of about 0.2g were ground in liquid nitrogen and added to 2ml/g extraction buffer, consisting of 30mM tris HCl (pH 7.5), 20mM NaCl and 1mM PMSF. Samples were left for 15 minutes at 4°C with shaking. Samples were then centrifuged at 10 000 rpm for 15 minutes at 4°C. The supernatants were heated for 10 minutes at 95 ° C and recentrifuged. The supernatant containing the heat-soluble protiens was retained. Protein content was calculated using a Bradford assay (Bradford, 1979). The remainder of the supernatant was concentrated by the addition of 4V acetone and left at –20°C overnight. The samples were then centrifuged at 15 000 rpm for 30 minutes and the acetone was poured off.

The samples were added to sample application buffer, heated at 95°C for 10 minutes and then run on a 20% PAGE gel for visualisation, which was stained with Coomassie blue for three hours and then destained (Laemmli, 1970).

Results and Discussion

A. Sugars

1. Dehydration

For *C.wilmsii* the levels of glucose in the fully hydrated tissue (85.5 ± 6.0 μmol g^{-1} DW) were greater than the fructose levels (21.3 ± 9.1 μmol g^{-1} DW) (Fig.1A). The higher level of glucose may be due to the fact that the only source of fructose would be from sucrose hydrolysis, whereas glucose can come from starch degradation (Whittaker *et al* 2001). There was an initial increase in fructose from 100% to 78% relative water content (RWC) followed by a significant decrease in the later stages of dehydration. The glucose values remain relatively constant throughout dehydration. This pattern is consistent with the findings of Whittaker *et al,* (2001), who report an initial increase in glucose and fructose in *Sporobolus stapfianus* and *Xerophyta viscosa* in the earlier stages of drying, possibly due to increased starch breakdown by amylase or by some initial hydrolysis of sucrose. In the early stages of dehydration respiration is maintained (Farrant, 2001) so sucrose may be hydrolysed initially to supply glucose and fructose for metabolism.

The relatively constant levels of glucose and fructose may be due to the fact that some *Craterostigma* spp are known to utilise 2-octulose, a product of photosynthesis as the main source for sucrose accumulation during dehydration (Norwood *et al,* 1999) and although 2-octulose utilisation has not been shown for *C.wilmsii,* if it is occuring, *C.wilmsii* is probably not therefore using significant levels of fructose and glucose for sucrose accumulation.

Sucrose levels in fully hydrated (100% RWC) *C.wilmsii* leaves were very low (Fig.2A), 20.1 ± 12.9 μmol g^{-1} DW; which is comparable to the levels of fructose, but at 78% there was a rapid 50-fold rise in sucrose levels which remained relatively constant until about 2% RWC where the concentration declined to 526.0 ± 111.9 μmol g^{-1} DW). But this level was still considerably higher than that of the hydrated leaves. Cooper and Farrant (2002) also found that *C.wilmsii* accumulated sucrose quite early on in the desiccation process. As a homiochlorophyllous species, this plant may need the protection offered by sucrose early on in dehydration, because there is a period before the accumulation of anthocyanins and leaf curling where the plant will be exposed to photosynthetically active radiation (PAR), that, in the absence of water can form damaging free radicals. The early accumulation of sucrose and vitrification may help to put a stasis on metabolism and protect structures from free radicals while the chloroplasts are still exposed to light. The fact that photosynthesis is not shut down in this plant could contribute to the rapid accumulation of sucrose.

In *Xerophyta schlecterii* glucose and fructose levels were consistently low in comparison to the accumulated sucrose levels. Fructose appeared to decline slightly at (19.7 ± 9.0 μmol g^{-1} DW (Fig.1B)). Glucose levels were also relatively constant but, as with the fructose, declined later in the later stages of dehydration to 12.2 ± 0.7 μmol g^{-1} DW (Fig.1B). *X.schlecterii* is very similar in size and morphology to *X.viscosa* and the fluxes of glucose and fructose during dehydration were similar in these two species. In *X.viscosa,* Whittaker *et al* (2001) report that glucose peaked at 78% RWC and declined thereafter, and fructose remained constant from 100% to 78% RWC before declining to the same level of glucose at 55% RWC. The glucose

and fructose levels in *X.viscosa* and *X.schlecterii* were much lower than those of *S.stapfianus* (Whittaker *et al,* 2001). This may reflect different rates of starch degradation or phosphorylation rates in these two *Xerophyta* species or possibly differences in sucrose cycling.

Sucrose concentration in *X schlecterii* was initially low (at about the same level as fructose and glucose). Due to high standard deviations it is not cleat that an increase occurred at 65% RWC (the standard deviation was 186.2 μmol g^{-1} DW). Levels did increase after further dehydration to 4% RWC (Fig.2B). The maximum concentration of sucrose in *X.schlecterii* was roughly 2 times lower than the maximum concentration in *C.wilmsii.* It is not known if *Xerophyta* spp utilise 2-octulose during dehydration as a sucrose source; it is possible that these species utilise a different but as yet unidentified source for sucrose that may be less abundant than 2-octulose, which may explain the lower levels of sucrose in dehydrated X*erophyta schlecterii and X.humilis* leaves. It is possible that these species rely on a source for sucrose that is non-photosynthetic, as these plants are poikilochlorophyllous and dismantle their chloroplasts during drying. Or it is possible that *Xerophyta* spp need less protection from sucrose due to the accumulation of other compatible solutes.

Whittaker *et al* (2001) reported that in *X.viscosa,* sucrose accumulated 2-fold from 89% to 55% RWC, and did not increase significantly below 55% RWC. *X schlecterii* and *X.viscosa* show a similar pattern of sucrose accumulation, which may be expected due to their physiological similarities. Acid invertase activity was found to be maintained in *X.viscosa* (Whittaker *et al,* 2001) during dehydration, possibly in

readiness for rehydration and rapid mobilisation of this stored sucrose, so further increases in sucrose accumulation may be limited by this activity.

Glucose and fructose in *X.humilis* exhibited similar trends during drying. Fructose was initially 72.2 ± 59.2 μmol g^{-1} DW and declined during from 100% to 80% RWC, after which levels remained low at around 5.8 ± 1.4 μmol g^{-1} DW until 56% RWC (Fig.1C). At 6% RWC levels increased slightly but remained low. Glucose also declined from 100% to 80% RWC, remaining constantly low for the remainder of the drying period (Fig.1C).

Sucrose accumulation only occurred late in dehydration in *X.humilis* (Fig.2C), unlike in *C,wilmsii*, where most of the sucrose accumulation occurred by the intermediate stages of drying. In *X.humilis* sucrose concentration in the leaves remained constant and very low until around 6%, where a peak of 284.4 ± 88.9 μmol g^{-1} DW was observed. It is not clear due to high standard deviations at 65% RWC whether *X.schlecterii* accumulates sucrose very early during drying or later in the drying process, so it is difficult to compare the timing of accumulation between the three species, but it is possible *X.schlecterii* accumulates sucrose later in drying like *X.humilis,* because a late peak at 4% RWC was observed after a low concentration at 34% RWC (Fig.2B).

Farrant *et al*, (2002, unpublished data) have found that *X. humilis* can accumulate sucrose efficiently when dehydrated in the dark and have suggested that in *X. humilis* sucrose is obtained by conversion from an alternative (as yet unknown) metabolite, that is independent of photosynthesis. This may explain the late accumulation of sucrose in this species. In species that utilise photosynthetic products

or storage reserves for sucrose synthesis, accumulation will occur in the early and intermediate stages of dehydration, before photosynthesis and respiration cease. But *X.humilis* may be relying on a source that is translocated or mobilised late in dehydration in both the light and in darkness, so its sucrose accumulation may be triggered later. This may be as a result of the fact that *X.humilis* is a poikilochlorophyllous resurrection plant, and shuts down photosynthesis during desiccation by disassembly of the chloroplasts, and so it must rely on a non-photosynthetic source for sucrose accumulation. Fructans, which are stored in grass species during periods of high light, and have been found to accumulate in dehydration-tolerant cultivars of wheat (Kepresi *et al,* 2002) could be a candidate for such a source in *Xerophyta* spp. Fructans are stored in the vacuoles, and are not directly dependent on photosynthesis for accumulation (Salisbury and Ross, 1992). Further investigation into the possible source of sucrose in these plants should be carried out.

The fact that *C.wilmsii* accumulates sucrose much more quickly than *X.humilis* may be related to the fact that it can still photosynthesise (as it is a homiochlorophyllous species) during the early and intermediate stages of drying, until anthocyanin accumulation and leaf curling are complete. And as it is possibly relying on a photosynthetic product for sucrose accumulation, large amounts will be available while the leaf is drying, until photosynthesis ceases, later in the dehydration process.

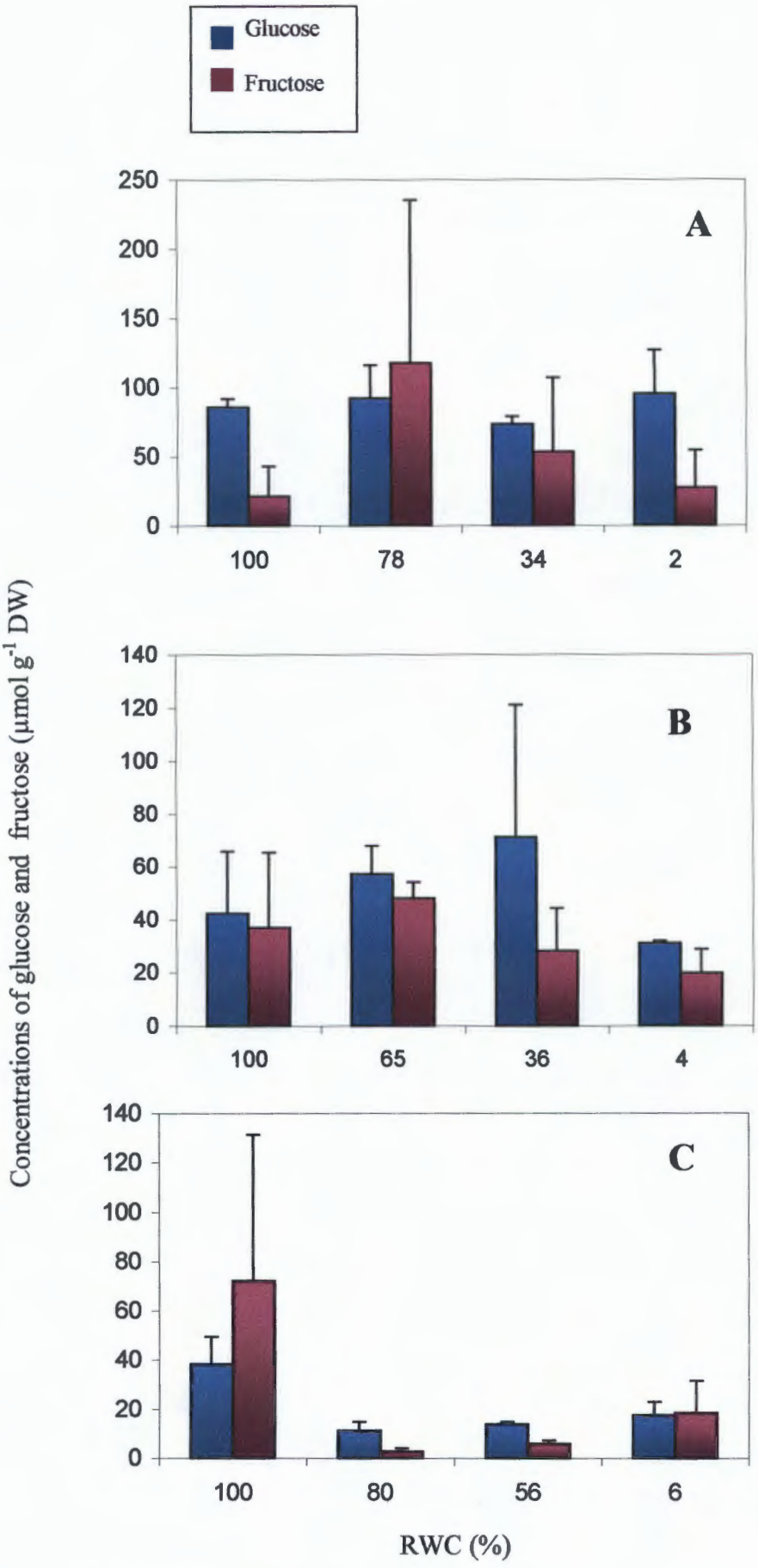

Fig.1. Concentrations of glucose and fructose in (A) *C.wilmsii,* (B) *X.schlecterii*, (C) *X.humilis* at different RWC %'s during dehydration. Values are the mean + SD of 1-3 assays per 1-3 extracts.

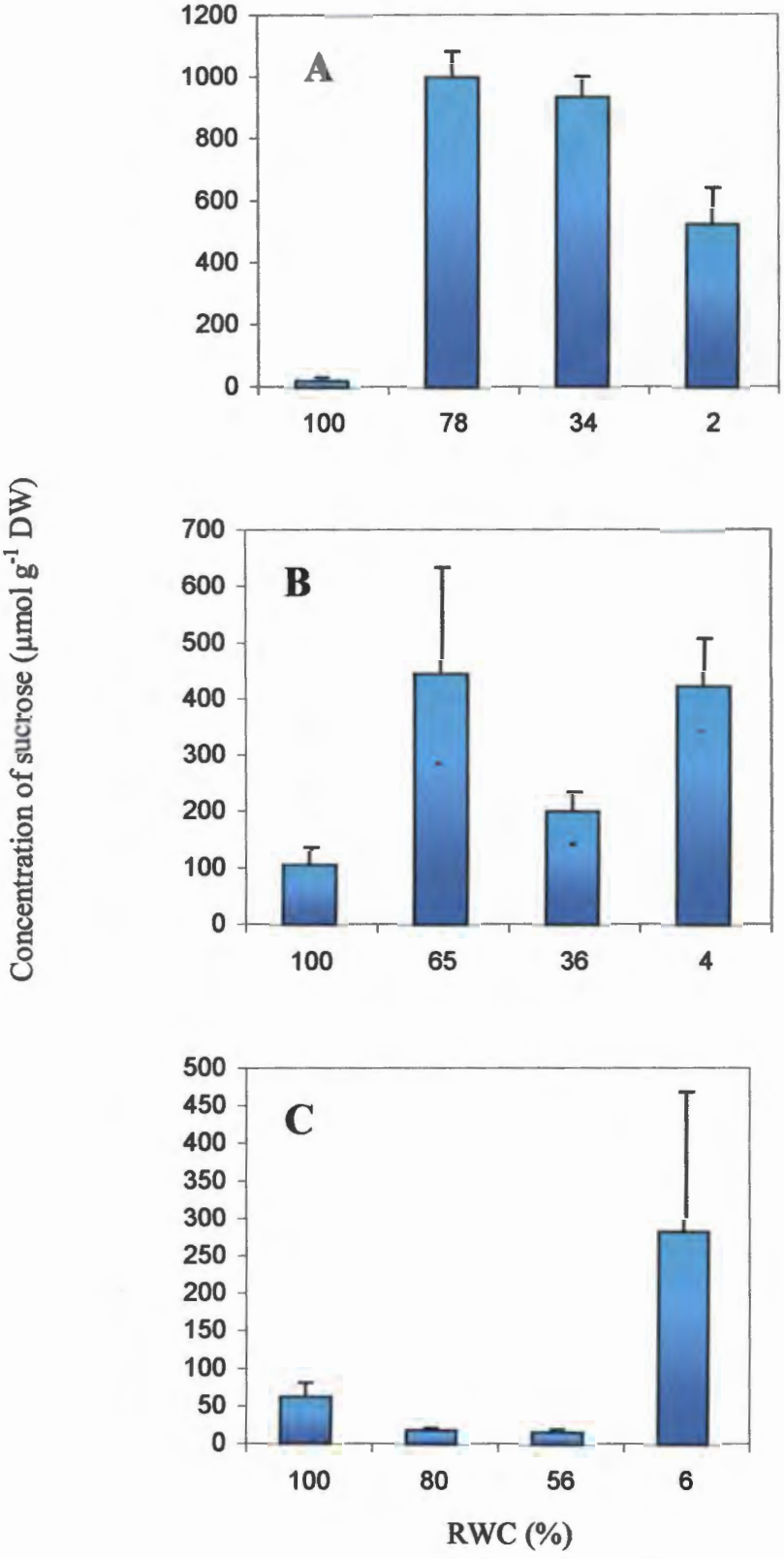

Fig.2. Concentrations of sucrose in (A) *C.wilmsii,* (B) *X.schlecterii*, (C) *X.humilis* at Different % RWC 's during dehydration. Values are the mean + SD of 1-3 assays per 1-3 extracts.

2. Rehydration

C. wilmsii exhibited a transient accumulation of fructose and glucose after 20 hours from initial rehydration, possibly due to rapid hydrolysis of the stored sucrose . Invertase activity was seen to increase in *S stapfianus* during rehydration (Whittaker *et al,* 2002) so it may be possible that this increase in fructose and glucose corresponds with an increase in invertase activity in *C. wilmsii*. These levels dropped off once full rehydration had occurred after 92 hours (Fig 3.A) to 62.3 ± 11.6 μmol g^{-1} DW for fructose and 51.8 ± 11.2 μmol g^{-1} DW for glucose. The low levels of glucose and fructose probably are due to the fact that they are being utilised by metabolism during rehydration. Sucrose concentration dropped 4-fold from 0 hours to 20 hours after rehydration (Fig 4A). This was the most significant decrease, so it appears that after 20 hours exposure to water, the plant has recovered its metabolism, and sucrose is being hydrolysed into fructose and glucose for respiration.

In *X. schlecterii* fructose and glucose levels remained relatively constant throughout rehydration (Fig.3B). It is possible that glucose and fructose produced by sucrose hydrolysis was immediately incorporated into metabolism and so transient accumulation was not detected after 20 hours. Sucrose concentrations did not drop as dramatically as in *C. wilmsii,* but did decrease progressively throughout the rehydration process. This plant is large, and rehydrates more slowly than *C.wilmsii,* so the enzymes involved in reinitiating metabolism will resume their activity later on a time scale, because they will only resume their activity once sufficient water is present in the cells for metabolic reactions to occur. This may explain the slower decline in sucrose levels. The final concentrations of sucrose in the leaves of *X.*

schlecterii, (310.7 ± 40.8 μmol g^{-1} DW, Fig.4B), were far higher than those in *C. wilmsii* (32 μmol g^{-1} DW, Fig.4A).

X.humilis fructose concentrations showed a slight decline at 20 hours after rehydration, but rose to original levels (24.4 μmol g^{-1} DW, Fig.3C) by 92 hours after rehydration. Glucose concentrations remained relatively constant throughout rehydration, with a slight decrease after initial rehydration (Fig.3C). Possibly the rate of sucrose breakdown and the rate of channeling of hexose sugars into metabolism are equal in *X. humilis.* Sucrose concentration progressively decreased during rehydration, indicating the increased hydrolysis of sucrose by invertase to yield glucose and fructose for rapid metabolism (Fig.4C)

B. Hexokinase

1. Dehydration

Overall, hexokinase activity was found to be much lower in all 3 species tested than reported for *X. viscosa* (Whittaker *et al,* 2001). In *C. wilmsii* initial activity in leaves of 100% RWC was 0.018 ± 0.014 μmol min^{-1} g^{-1} DW. There was a slight increase in activity as leaves were dried to 34% RWC, with a large 5-fold increase at 2% RWC to 0.269 ± 0.018 μmol min^{-1} g^{-1} DW (Fig.5A). There was not an increase in hexokinase activity at the period of highest sucrose accumulation (at around 78% RWC) (Fig.2A),

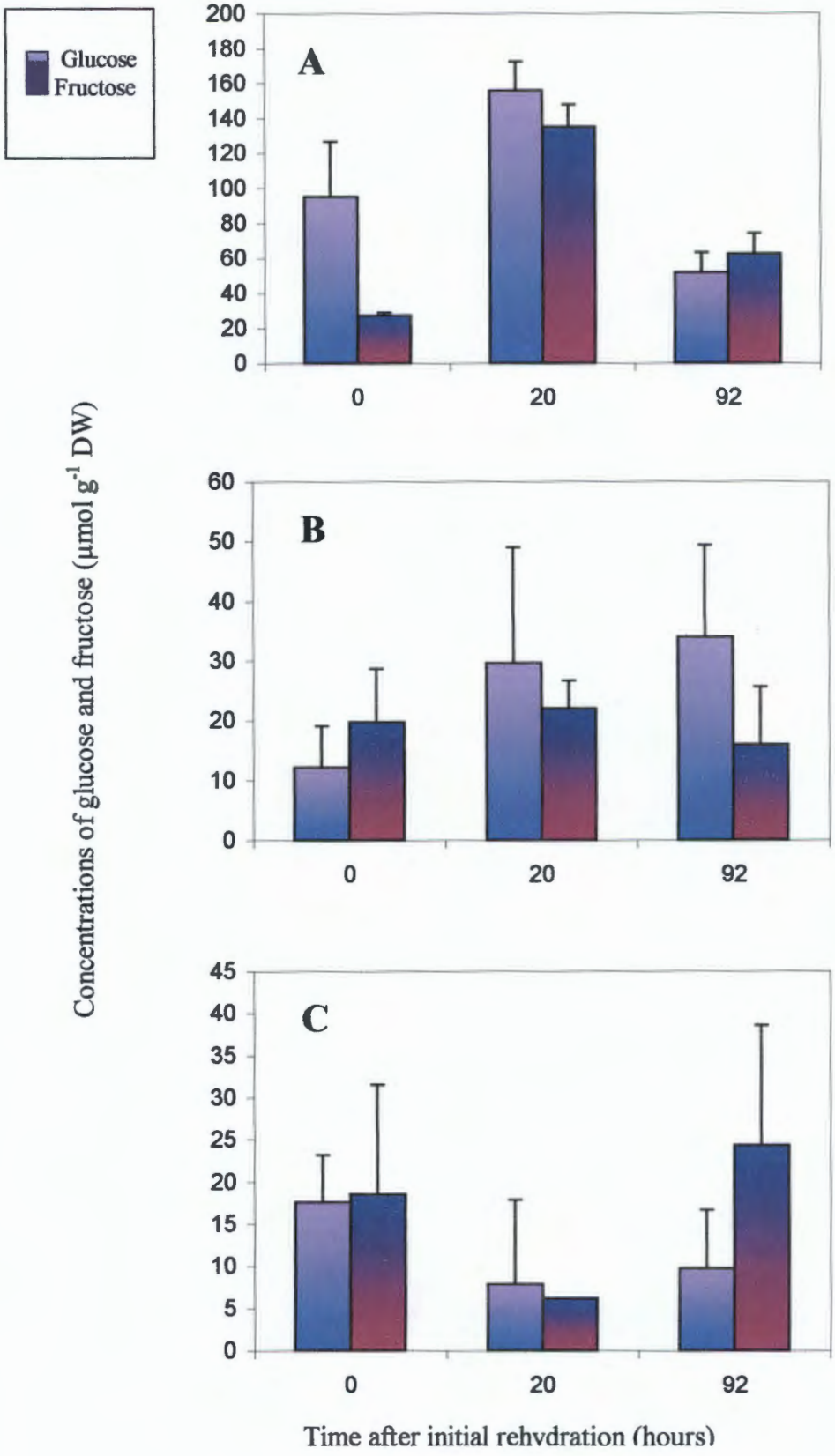

Fig.3. Concentrations of glucose and fructose in (A) *C. wilmsii,* (B) *X. schlecterii*, (C) *X. humilis* with increasing time from initial rehydration. Values are the mean + SD of 1-3 assays per 1-3 extracts.

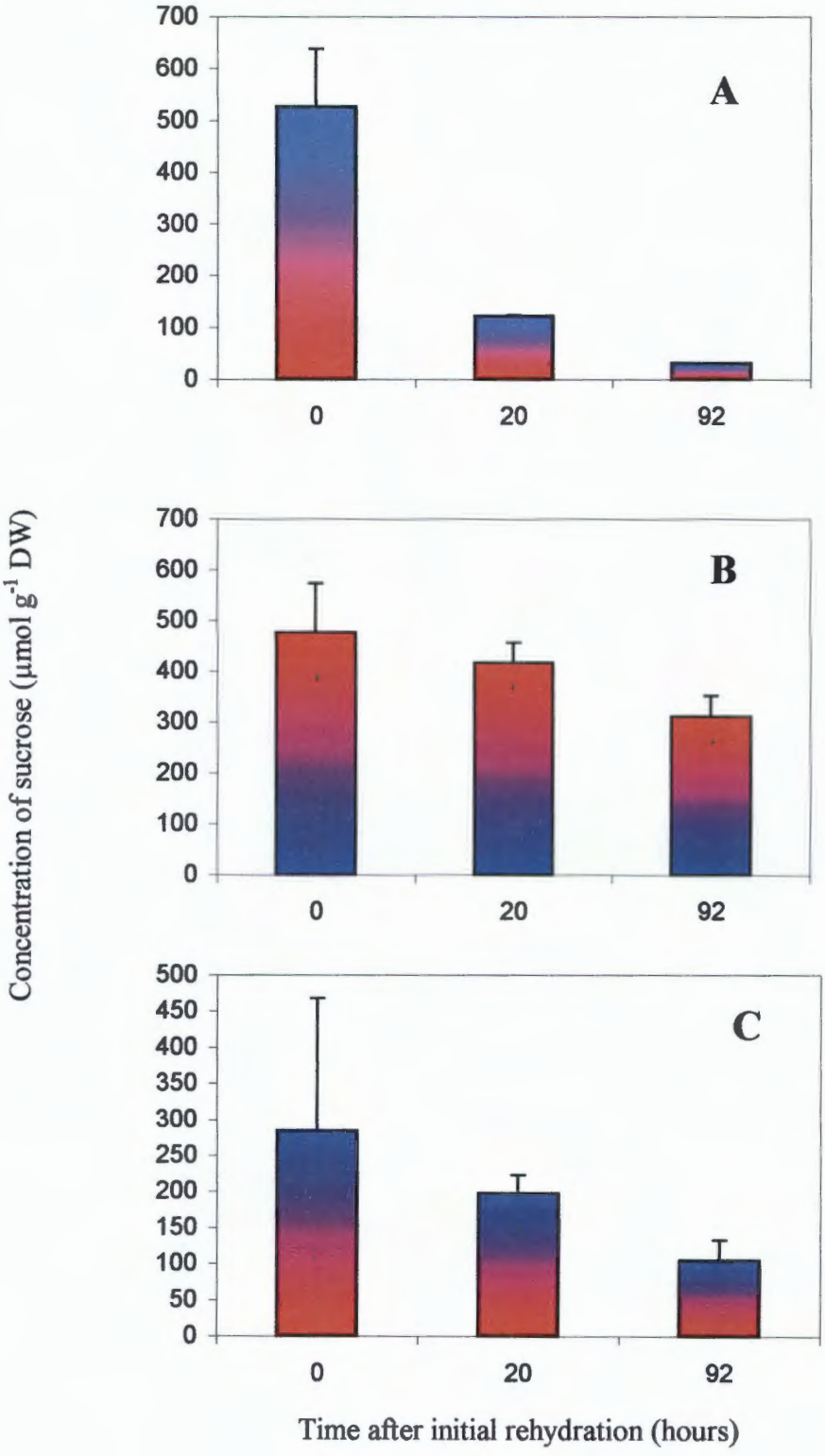

Fig.4. Concentrations of sucrose in (A) *C. wilmsii,* (B) *X. schlecterii,* (C) *X. humilis* with increasing time from initial rehydration. Values are the mean + SD of 1-3 assays per 1-3 extracts.

which implies that hexokinase is not a major enzyme involved in sucrose accumulation in this species.

It may be possible that the large increase in hexokinase activity so late in dehydration may be a preparative step for rehydration, when large amount of glucose and fructose will be made available from the stored sucrose hydrolysis to be channelled into metabolism (Whittaker *et al,* 2001). As *C.wilmsii* is a homiochlorophyllous plant, it recovers metabolism rapidly after initial rehydration, and this could necessitate enzymes involved in metabolism, such as hexokinase, to be on standby.

In *X. schlecterii* the hexokinase activities were low in comparison to those of *C.wilmsii*; with maximal levels of 0.05 ± 0.006 μmol min^{-1} g^{-1} DW occurring between 65% and 36% RWC (Fig.5B). This slight peak may be to ensure that biosynthesis of sucrose (and the supply of hexose phosphates by hexokinase for this synthesis) exceeded the hydrolysis by acid invertase that was found to have constant activity in throughout dehydration in *X. viscosa* and *S. stapfianus* (Whittaker *et al,* 2001). The fact that the glucose, fructose and sucrose concentrations in *X.schlecterii* remained constant over the period of the slight peak in hexokinase activity may support this.

The highest level of activity observed in *X.viscosa* by Whittaker *et al* (2001) was about 1.6 μmol min^{-1} g^{-1} DW at about 30% RWC. The considerably lower activities in *X. schlecterii* are strange considering the similarities in their sugar fluxes during dehydration (as outlined above). It is possible that these species differ in their utilisation of hexokinase during dehydration.

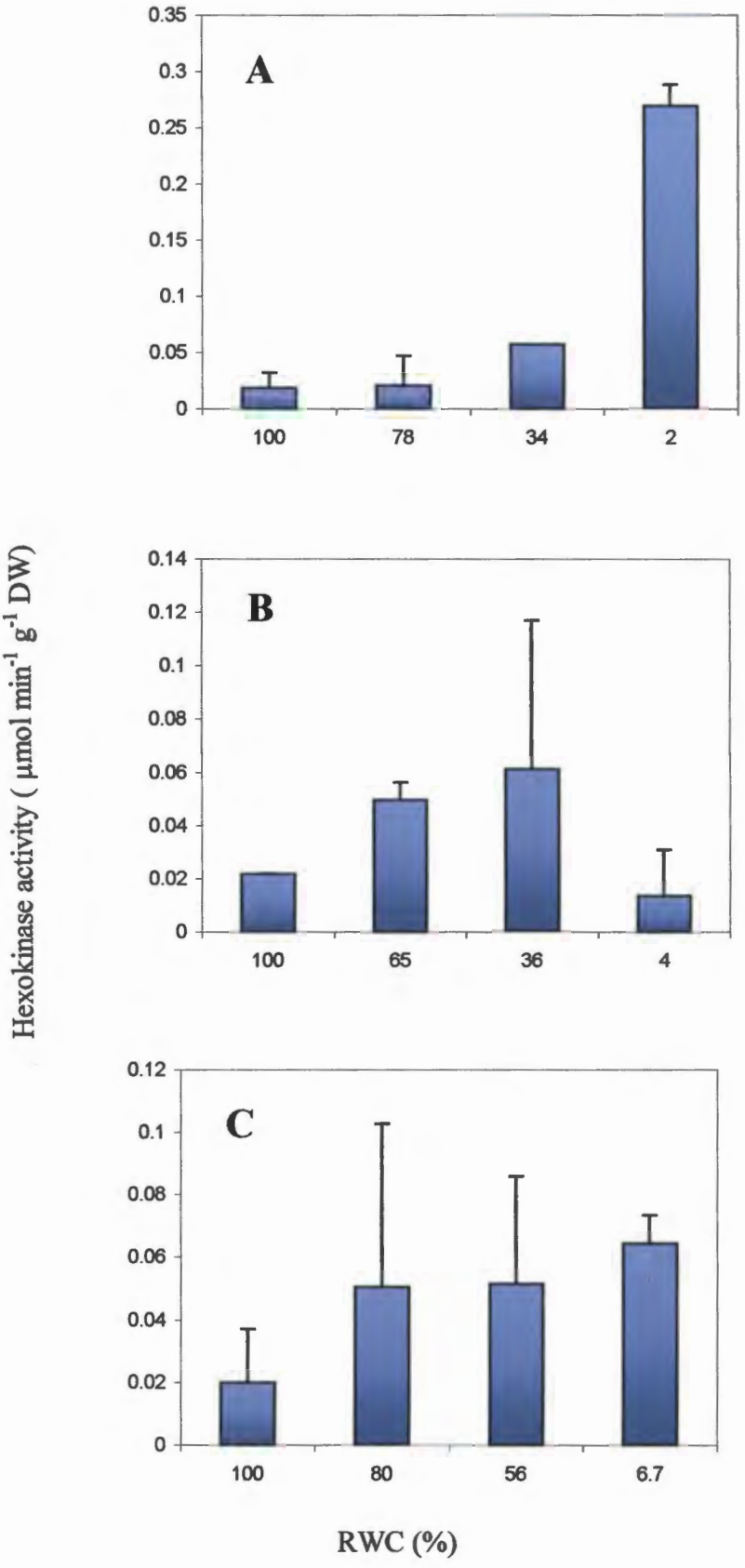

Fig.5. Activity of hexokinase in (A) *C. wilmsii,* (B) *X. schlecterii*, (C) *X. .humilis* at different RWCs during dehydration. Values are the mean + SD of 1-3 assays of each of 1-3 extracts.

Hexokinase activity in *X. humilis* was again found to be very low in comparison to *X. viscosa*. Activity increased from the hydrated to the dehydrated state (Fig 5C), which again may be due to preparation of the leaf cells for rapid channelling of glucose and fructose into metabolism during rehydration. This species exhibited a late accumulation of sucrose that correlates with the late increase in hexokinase activity, so hexokinase may be involved in the sucrose accumulation of this species. However, the levels of glucose and fructose remained relatively constant throughout this period, so it is not clear that these hexose sugars are being used as substrates for sucrose accumulation.

2. Rehydration

In the rehydration experiments performed on *S. stapfianus* by Whittaker *et al,* (2002), hexokinase was found initially to decrease slightly but to increase again at 100% RWC.

In *C. wilmsii*, the levels of hexokinase activity dropped 3-fold from the dehydrated state to 20 hours after rehydration (Fig6A). By 20 hours of rehydration the sucrose hydrolysis in this plant appeared complete (Fig.4A), so it would be interesting to know if the levels of hexokinase were maintained from 0-20 hours rehydration, and then decreased from then because sucrose hydrolysis was complete. A more detailed rehydration study with more sampling time points would be able to verify this.

The standard deviations of activity of hexokinase in *X. schlecterii* were very variable throughout rehydration, but activity appeared to remain relatively constant (Fig.6B). The sucrose levels did not drop as dramatically in this species as in *C. wilmsii (*refer to Figs. 4A and B), and the levels of hexokinase activity are considerably lower with a peak of 0.02μmol min^{-1} g^{-1} DW (Fig.6B), as opposed to 0.07μmol min^{-1} g^{-1} DW (Fig.6A) in *C. wilmsii.* Hexokinase may not need to be as active in *X. schlecterii* into feed hexose sugars into metabolism to prevent accumulation of the hexose sugars and possible negative feedback inhibition of enzymes. This plant is larger than *C.wilmsii* and rehydrates more slowly, and as a result metabolism resumes later than in *C.wilmsii*. This may explain the lower rates of hexokinase activity.

In *X. humilis* the activity of hexokinase dropped slightly from 0 to 20 hours rehydration, and again from 20 to 92 hours rehydration. The activities were low throughout (Fig.6C), relative to *C.wilmsii* (Fig.6A). Hexokinase activity was observed to drop in *S. stapfianus* during rehydration (Whittaker *et al,* 2002). It may be that as sucrose is hydrolysed, the initial higher activities of hexokinase possibly deal with the initial high rate of glucose and fructose production, by phosphorylating them so that they can enter metabolism. Once much of the sucrose is already hydrolysed (later in the rehydration process) hexokinase activity will not be as important, and may be downregulated.

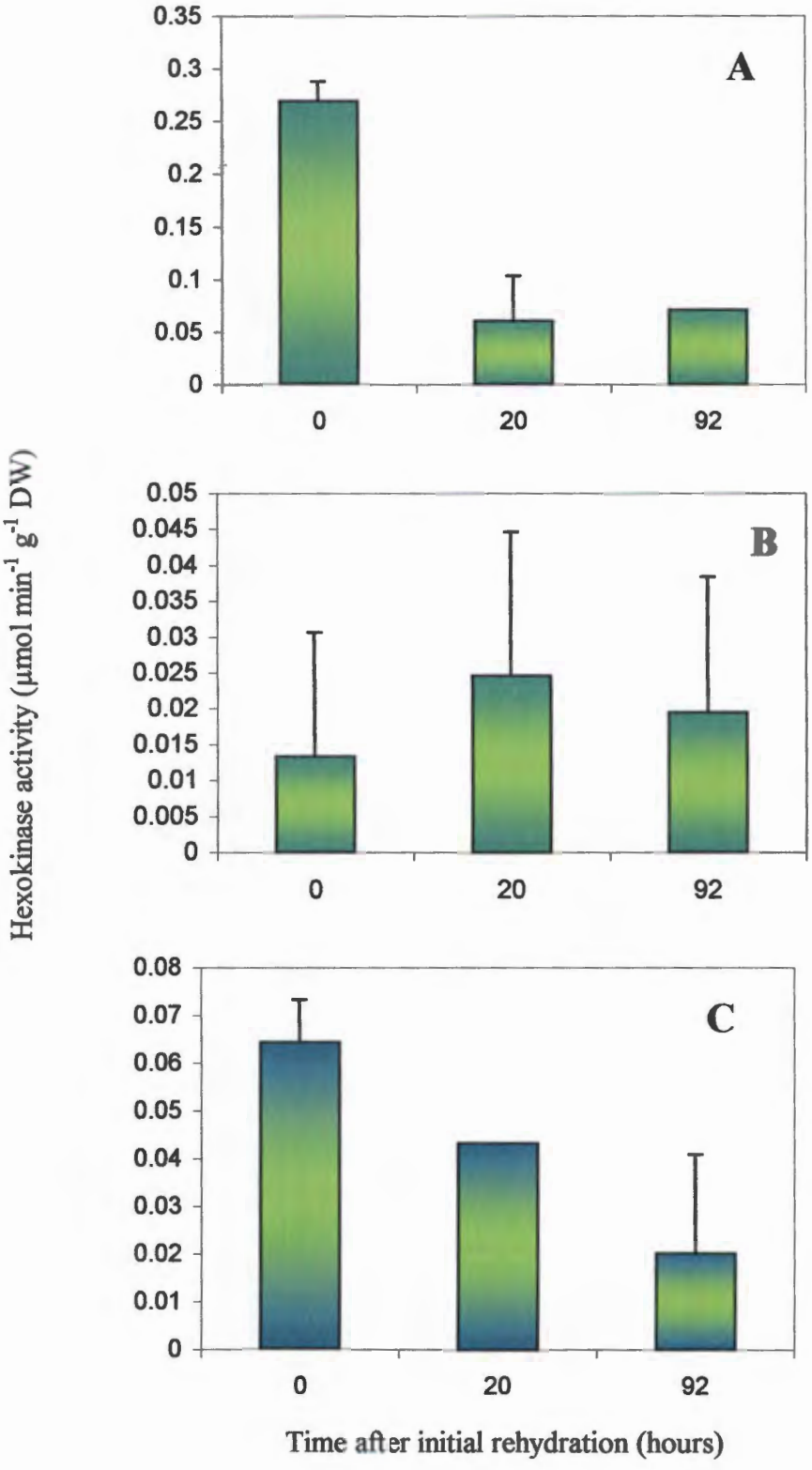

Fig.6. Activity of hexokinase in (A) *C.wilmsii,* (B) *X.schlecterii,* (C) *X.humilis* at different times during rehydration. Values are the mean + SD of 1-3 assays of each of 1-3 extracts.

C. LEA-like proteins

Due to a shortage of sample, *C.wilmsii* was not presented in the final gels, although preliminary gels gave evidence of two heat-soluble proteins in *C.wilmsii* that were present in both hydrated and dehydrated samples (data not shown).

There was evidence of the presence of LEA-like or heat-soluble proteins in the three species presented (Figure 9A an B). These proteins were most clearly visualised in the dehydrated *X.schlecterii* extract (Fig.9A and B, lane 5). There was evidence of a protein common to all 6 lanes between the H1 and H3 histone markers. It was a faint band but more clearly visible on the actual gels and not clear in the scanned gels. This band was clearest in Fig.9A. It appears that this is a common constitutive heat-soluble protein, that may have a function in unstressed cells.

In *X.viscosa,* there is a protein band between the 13.7 kDa and 11.2 kDa markers (lane 2, Fig.9A and B) that is not present in the dehydrated sample, and there is a protein band of larger molecular weight (it has migrated a shorter distance) that roughly corresponds to the 13.7 kDa marker in the dehydrated sample (lane 3, Fig.9A and B), that is not present in the hydrated sample. It appears that a heat-soluble protein is upregulated during water stress in this species, and another is not needed during dehydration. The protein that was upregulated may have a specific function such a membrane or macromolecule integrity or may be involved in glass stabilisation.

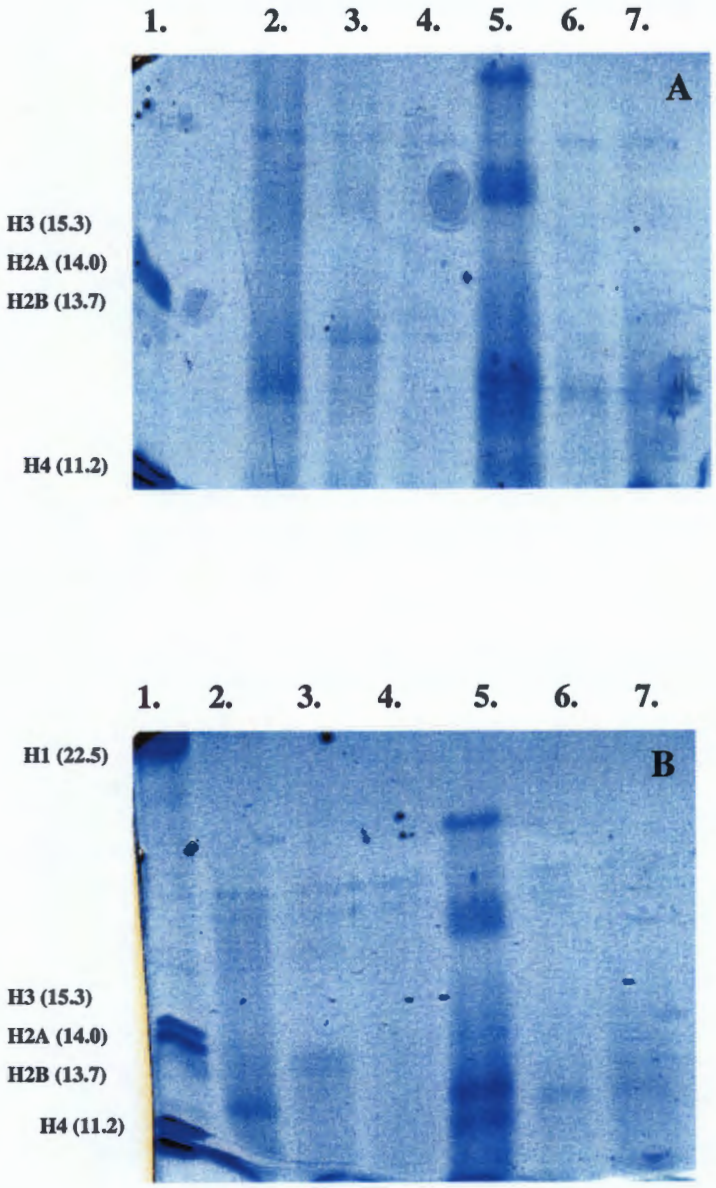

Figure 9. A and B are 20% PAGE gels of the heat soluble proteins (2) hydrated *X.viscosa,* (3) dehydrated *X.viscosa,* (3) hydrated *X.schlecterii,*(5) dehydrated *X.schlecterii,* (6) hydrated *X.humilis* and (7) dehydrated *X.humilis.*Lane 1 in both figures is a chicken histone marker (molecular weights in kDa on the side of the figures).

In *X.schlecterii,* the visibility of the bands of the hydrated sample (lane 4) was very poor, and it is difficult to comment on the presence of proteins other than the band of relatively high molecular weight that was present in all species. The dehydrated sample of *X.schlecterii* (lane 5) had 4 clear bands other than this common protein. Two band of higher molecular weight were found between the 22.5 kDa and the 15.3 kDa markers, with one band being larger than the common protein and one being smaller (having migrated further). Two bands were seen between the 13.7 kDa and the 11.2 kDa markers (Fig.9B). It is not possible to comment on whether this species upregulates these proteins during stress, due to the poor staining of the hydrated sample, but it is evident that heat-soluble or LEA-like proteins are present in *X.schlecterii.*

Apart from the protein band that is common to all three species (clearest in Fig.9A), there was evidence of one other protein that was common to both the hydrated and dehydrated samples of *X.humilis* (lanes 6 and 7). It appears that this species does not induce LEA-like proteins specifically during dehydration stress, but has constitutive proteins that function in unstressed cells as well as in dehydrated cells.

Conclusions

The glucose and fructose levels in all three species examined were relatively constant throughout dehydration. All three species accumulated sucrose, which is consistent with the literature on desiccation tolerant angiosperms (Cooper and Farrant, 2002, Scott, 2000, Whittaker *et al,* 2001). However the timing of the sucrose accumulation varied. The homiochlorophyllous *C.wilmsii* accumulated sucrose far earlier than the poikilochlorophyllous *X.humilis.* This may be related to the source for sucrose that they are utilising, or to the necessity for protection at different stages in dehydration. *X.humilis* may be relying on a sucrose source that is mobilised late in dehydration, and that is not produced by photosynthesis. This is further suggested by the findings of Farrant *et al,* (2002, unpublished), who have found that *X.humilis* can accumulate sucrose when dried in the dark. *C.wilmsii* is possibly utilising 2-octulose, a photosynthetic reserve, for its sucrose accumulation. This would be used up early in dehydration, as photosynthesis is shut down later in drying.

None of these species seemed to rely on glucose and fructose being incorporated into sucrose via phosphorylation by hexokinase. In *C.wilmsii*, these constant levels may indicate that glucose and fructose are not important substrates for sucrose accumulation, because a large increase in sucrose at 78% RWC was not accompanied by a large decline in glucose or fructose (Fig.1A). 2-octulose may be playing a more important role in the sucrose accumulation of this species. Hexokinase activity did not increase in this species during the period of sucrose accumulation (between 100% and 78% RWC, Fig.2A), so this further suggests that glucose and fructose are not being channelled into sucrose biosynthesis via phosphorylation by

hexokinase. Hexokinase activity did increase late in dehydration (Fig.5A), indicating that it is present in preparation for rehydration, where metabolism will be resumed.

In *X.schlecterii* and *X.humilis*, glucose and fructose levels were relatively equal and constant throughout dehydration, with a slight decline in both sugars in *X.humilis.* In *X.schlecterii,* an increase in sucrose (from 100% to 65% RWC, (Fig.2B)) was not accompanied by a decline in glucose or fructose (Fig.1.B). It was not clear exactly when sucrose increased in this plant due to high standard deviations. Hexokinase activity did exhibit a slight peak that may be important to ensure that the supply of hexose phosphates into metabolism and biosynthesis exceeds their production by invertase hydrolysis (Fig.5B). The activity of hexokinase did not clearly increase in *X.humilis* (although the high standard deviations of the data make it difficult to predict a trend) until late in dehydration (Fig.5C). This was similar to *C.wilmsi;* it appears these two species prepare for rehydration with an increase in hexokinase activity.

During rehydration desiccation tolerant plants are able to re-initiate metabolism and cell functioning. In *S. stapfianus* no build up of hexose sugars was observed during rehydration (Whittaker *et al,* 2002). This suggests that they are being incorporated into primary carbon metabolism at the same rate as they are being produced by sucrose hydrolysis. Photosynthesis will be more rapidly resumed in the homiochlorophylous *C.wilmsii* than in the two poikilochlorophyllous *Xerophyta* species as the latter will have to resynthesise their photosynthetic apparatus *de novo* during rehydration.

C.wilmsii exhibited a transient accumulation of glucose and fructose at about 20 hours after rehydration (Fig.3A) but *X.schlecterii* and *X.humilis* exhibited relatively constant glucose and fructose concentrations (Fig.3B and C). Invertase activity may be exceeding hexokinase activity in *C.wilmsii,* which was in fact observed to decline after 20 hours of rehydration (Fig.6A). In the two poikiloclorophyllous monocotyledonous species, hexokinase activity remained relatively constant throughout rehydration, with a slight decline in *X.humilis* in the final stages of rehydration (Fig.6C). The drop in sucrose concentration was much larger in *C.wilmsii* than in the two poikilochlorophyllous species (Figs.4A-C). The recovery of these plants is expected to be slower, and so the sucrose may be needed to protect membranes and macromolecules, and stabilise glasses for longer.

It appears that hexokinase does not play as an important role in the three species investigated here, as it does in *X.viscosa* and *S.stapfianus* (Whittaker *et al*, 2001). Glucose and fructose do not appear to be major sources of substrate for sucrose biosynthesis in any of these three species, they all appear to be utilising another source for sucrose accumulation.

LEA-like proteins were found to be present in all species tested, but the poor visibility of the gels made it difficult to comment on the amounts of protein present and to compare between hydrated and dehydrated samples. These proteins do appear to be present during dehydration in all the species, but as to whether they were produced specifically induced by dehydration will require further investigation. A protein common to all species was observed, with a relatively high molecular weight. This protein may a function that is important in all plants, or only in desiccation

tolerant plants. Further investigation into the identities of these proteins and their functions is required.

Further investigation

A further indication of hexokinase activity would be the levels of fructose and glucose-6-phosphate in the tissues of these three plants. Invertase activity could also be tested to establish whether it exhibits activities similar to those already reported for *X.viscosa* and *S.stapfianus* by Whittaker *et al* (2001 and 2002) during rehydration and dehydration.

The presence of 2-octulose in *C.wilmsii* leaves would be an important investigation to confirm that the source for sucrose accumulation in this species is the same as that reported for *C.plantagineum* by Norwood *et al* (1999). The activities of 2-octulose biosynthetic enzymes could also be measured. It would be useful to identify any unknown sources for sucrose accumulation in *X.humilis* and *X.viscosa.*

A more detailed rehydration curve for all three species could be produced, to see exactly when sucrose begins to decline, and if this correlates with changes in any enzyme activity.

Finally, better and clearer gels should be run for all four species, and western blots, using antibodies for some LEA-like proteins should be carried out to identify

the proteins present in each species. A more detailed gel with more dehydration points and some rehydration points would give insight as to when certain proteins are induced.

Acknowlegements

I would like to thank my supervisor Prof. Jill Farrant for all her advice and guidance, Zeenat Rojoa for lots of help with experimental procedures, and Dr.Claire Van der Willigen, Keren Cooper, and Dr.Brigitte Hamman for technical advice. Thank you also to UCT and the Botany department for funding.

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